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UCRL-PROC-207711

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November 4, 2004

SPIE's 49th Annual Meeting
Denver, CO, United States
August 2, 2004 through August 6, 2004

Application of SERS Nanoparticles for Intracellular pH Measurements

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ABSTRACT

We present an alternative approach to optical probes that will ultimately allow us to measure chemical concentrations in microenvironments within cells and tissues. This approach is based on monitoring the surface-enhanced Raman scattering (SERS) response of functionalized metal nanoparticles (50-100 nm in diameter). SERS allows for the sensitive detection of changes in the state of chemical groups attached to individual nanoparticles and small clusters. Here, we present the development of a nanoscale pH meter. The pH response of these nanoprobes is tested in a cell-free medium, measuring the pH of the solution immediately surrounding the nanoparticles. Heterogeneities in the SERS signal, which can result from the formation of small nanoparticle clusters, are characterized using SERS correlation spectroscopy and single particle/cluster SERS spectroscopy. The response of the nanoscale pH meters is tested under a wide range of conditions to approach the complex environment encountered inside living cells and to optimize probe performance.

INTRODUCTION

Recent research has shown that chemical microenvironments play a crucial role in both normal and diseased tissues. Measuring chemical concentration variations or chemical gradients across membranes is important for understanding basic biological processes, and relevant to developing disease treatments. High spatial resolution is essential for measuring chemical concentrations in small microenvironments such as cells. In order to understand the role of these chemical microenvironments, we need tools that are capable of providing chemically specific information along with sufficient spatial resolution. Much of the data on chemical microenvironments was acquired using magnetic resonsance imaging. For example, magnetic resonance spectroscopy (MRS) has been used extensively to measure the intracellular and extracellular pH of tumors in cell-based studies as well as *in vivo*, monitoring tumor development in mice (Gillies, Raghunand et al. 2002). While MRS has been useful in identifying the presence of chemical microenvironments, the spatial resolution is limited to 0.1 to 1 mm. It is speculated that microenvironments are present on a much smaller scale prompting the need for higher resolution techniques for measuring chemical concentrations in these microenvironments.

Optical probes have been developed that can provide spatial resolution on the order of a micron. These probes are based on a variety of fluorescent dyes that change their fluorescent properties in response to chemical changes in their environment. For example, the dye fluorescein exhibits a sensitivity to pH, which changes both fluorescence lifetime and the spectral emission of the dye (Martin and Lindqvist 1975). These dyes, however, have a number of shortcomings: organic dyes rapidly photobleach under continuous excitation, limiting the observation time to typically about a few minutes. Also, the fluorescent dyes are generally used at very high concentrations in order to detect the signal over the autofluorescence

background of the cell. This can significantly alter the chemistry within cells, and increases the risk of toxicity of the fluorescent dyes.

Raman Spectroscopy uses specific, narrow lines caused by molecular vibrational modes to identify chemical groups and their states (Lewis and Edwards 2001). This is a great benefit for chemical identification; however, Raman signals are too weak to detect on the single-molecule or single-particle level. Also, there is no ability to select for particular analytes: in a heterogeneous environment, many molecules will contribute to the Raman signal. However, if the chemical group of interest is near a metal surface with a roughness on the nanometer scale, an enormous enhancement of the signal occurs, resulting in detectable signals from single particles. This phenomenon is known as Surface-Enhanced Raman Spectroscopy (SERS) (Pettinger, Tadjeddine et al. 1979). If the excitation is resonant with an electronic transition in the molecule, then Raman signals from single molecules may even be detected (Kneipp, Wang et al. 1997).

In order to overcome the limitations of MRS, fluorescence spectroscopy, and Raman spectroscopy for monitoring analyte concentrations in heterogeneous environments, we are currently developing a new generation of intracellular probes based on the surface-enhanced Raman scattering (SERS) of analyte-sensitive molecules attached to gold or silver nanoparticles. These nanoparticle probes will enable us to monitor the analyte concentration inside the highly heterogeneous chemical environment of cells and tissues with diffraction limited spatial resolution for extended periods of time. The signal strength allows detection of single particles or clusters, and the signal show little or no photodegradation with time. Individual nanoparticles/clusters can be imaged with confocal microscopy to obtain position information with diffraction-limited resolution. Moreover, the location of the nanparticles within the cell can be pinpointed to within 10nm by fitting the intensity profile (Michalet, Lacoste et al. 2001).

EXPERIMENTAL

Ag-colloid nanoparticles were prepared following the citrate reduction protocol from (Lee and Meisel 1982). The nanoparticles were subsequently functionalized with 4-mercaptobenzoic acid (4-MBA) (Aldrich) by adding a 30 μL aliquot of a 30 mM methanol solution of 4-MBA to the aqueous nanoparticle solution. This concentration of 4-MBA ensures that there is an excess of 4-MBA resulting in full coverage of each nanoparticle.

In the first set of experiments, nanoparticles were immobilized on a silanized glass coverslip (3-aminopropyltrimethoxysilane, Aldrich) and inserted into a custom designed flow chamber. The flow chamber was placed on a closed-loop X-Y piezo stage (Physik Instrumente) which was mounted on an inverted microscope (Zeiss, Axiovert S100TV). A helium-neon laser (632.8 nm, Research Electro Optics, LHRP-0501) was coupled into the back of the microscope and directed into a high numerical aperture oil immersion objective (Zeiss, Plan-Achromat 100X, 1.4 NA) that focused the laser to a diffraction limited spot on the sample surface. The light scattered by the particles was collected through the same 100X objective and focused onto a 100 μm pinhole to reject out of focus light. The Rayleigh scattered light was removed using a holographic notch filter (Kaiser Optical) and the remaining Raman scattered light was focused onto an avalanche photodiode (PerkinElmer, SPCM-AQR-14). Commercial scan control electronics and software (Digital Instruments, Nanoscope IIIA) were used to scan the stage over the focused laser in a raster fashion to generate an image of the sample surface. Once a particle was located in the image, it was positioned in the laser focus and the Raman scatter was directed into a spectrograph (SpectraPro 300i) equipped with a back-thinned LN cooled CCD camera (Roper Scientific, Spec-10:100B(LN)). SERS spectra with high signal-to-noise ratios were obtained with an integration time of 30 s and a laser power density between 1-10 kWcm-2.

For the remaining experiments, the nanoparticles were allowed to freely diffuse in solution while being monitored using confocal microscopy (Nikon TE300). The same helium-neon laser mentioned above was coupled into the back of the microscope and focused using a high numerical aperture oil immersion objective (Nikon, CFI PLAN APO 60X DIC). Scattered light was collected by the same objective and focused onto a pinhole (150 μm) to exclude out-of-focus light. A dichroic mirror (Omega Optical, 690 DRLP) split the emission between two Raman lines, and bandpass filters (Omega Optical, 695DF6 and

679DF6) excluded light away from these lines. The filtered light was then focused onto two avalanche photodiodes (PerkinElmer, SPCM-AQR-14). Each detected photon was timed using a counter-timer plug-in board (National Instruments, PCI-6602). Data acquisition and subsequent analysis were performed using home-built software using LabVIEW (National Instruments) and C++ (Microsoft Visual Studio .net 2002).

RESULTS AND DISCUSSION

Development of nanoparticle optical probes

The basic idea behind our nanoparticle optical probes is relatively straightforward and illustrated in Figure 1. Individual gold or silver colloids are coated with analyte-sensitive molecules such that the surface of the particles is fully saturated. Full surface coverage is important because, as mentioned above, SERS is an extremely sensitive effect and any adsorption of other molecules to the surface of our nanoparticle probes will lead to the occurrence of additional peaks in their Raman spectrum. The SERS response of these coated nanoparticles is unique and remains preserved even in the highly heterogeneous chemical environment of cells. Only changes in the conformation or the electronic properties of the probe molecules will lead to changes in their Raman spectrum. Such changes can be invoked by specific binding to the end groups of the probe molecules or other structural rearrangements, to which SERS is highly sensitive. This is illustrated in Figure 1b for the case of a binding event.

We have recently developed silver nanoparticles that are functionalized with mercaptobenzoic acid groups whose characteristic Raman spectra change as the groups are protonated and deprotonated (see Figures 1 & 2). This allows pH measurements in microenvironments of the size of the nanoparticles. Damage to the particles over time is minimal, and the signal is easily distinguished from background signal. This development can provide a way to measure the local pH inside different parts of a cell for extended periods of time, and to compare it with extracellular environments.

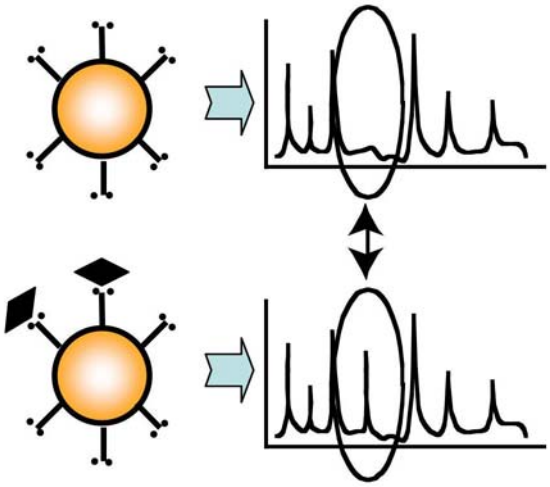

Figure 1: Schematic illustration of how nanoparticle probes can be used to monitor changes in the concentration of intracellular metabolites. When the SERS particles are labeled with specific probe molecules, changes in the SERS spectrum indicate binding of the probe molecule to the target analyte.

The development of new methods for long-term detection of pH in microenvironments will allow the effects of chemotherapeutic and radiotherapeutic agents to be monitored for greater lengths of time, with greater spatial resolution than previously possible. The effects of therapies on regions with specific pH gradients can be measured over extended periods of time. Also, if a specific fluorescent or SERS tag is developed for the specific agent in question, the uptake of the agent can be correlated directly with pH gradient.

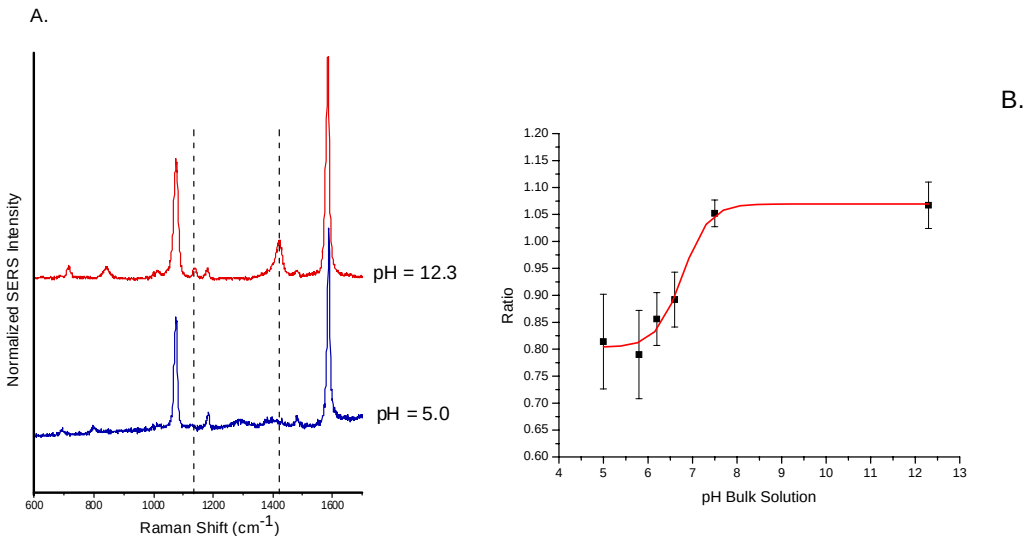

Figure 2 Example of a single silver nanoparticle, coated with mercaptobenzoic acid (MBA) in a pH-probe application. Changes in the local pH protonate/deprotonate the carboxylic acid group, which leads to characteristic changes in their Raman spectrum (A) as indicated by the dashed lines. The characteristic pH response of the MBA coated nanoparticles is shown in (B).

This is not the only possible application of these nanoparticles. Other chemical groups may be attached, allowing the detection of concentrations for many different chemicals, such as glucose, ions, CO_2, O_2, proteins, etc. The ability to measure chemical concentrations in different microenvironments within and near cells may prove crucial to understanding many biological processes since cells often use membranes to maintain chemical gradients intrinsic to function.

Before the SERS nanoparticle pH measurements can be reliably applied to new biological questions, additional technical improvements are required. Complications with the nanoparticles include the following: (1) different shapes and aggregation of the nanoparticles affect the signal strength (sharp edges produce the strongest signal), (2) ionic strength of the solution seriously affects the midpoint of the pH calibration curve for measurements (surface effects), (3) different shapes and particle aggregation may also affect the ionic sheath, and thus the pH calibration curve.

Characterization of pH response in bulk solution.

The pH is obtained from the SERS signal by calculating the ratio of a Raman line dependent on pH to a Raman line independent of pH. We have developed a rapid assay that focuses only on the relevant lines, allowing higher throughput in the number of particles analyzed (Figure 3). Fast pH measurements are tested by scanning confocal beam in solution containing MBA-coated silver nanoparticles. For each "burst" of photons (approximately 20 ms long), the ratio of the photons detected from the pH-variable line to the total number of photons detected is calculated, and placed in the histogram to the right. There is a clear shift in this ratio when the pH of the solution is changed from pH 8.5 to pH 5.5. The widths of the peaks in the histograms are similar to those found using the full spectroscopy in Figure 2.

Figure 4 shows results of these measurements, demonstrating the dependence of the ratio on pH The widths of the peaks demonstrate present resolution limits in our ability to determine the pH. We are now investigating whether different aggregation states of the nanoparticles, or different types of nanoparticles can narrow these peaks, increasing the pH resolution.

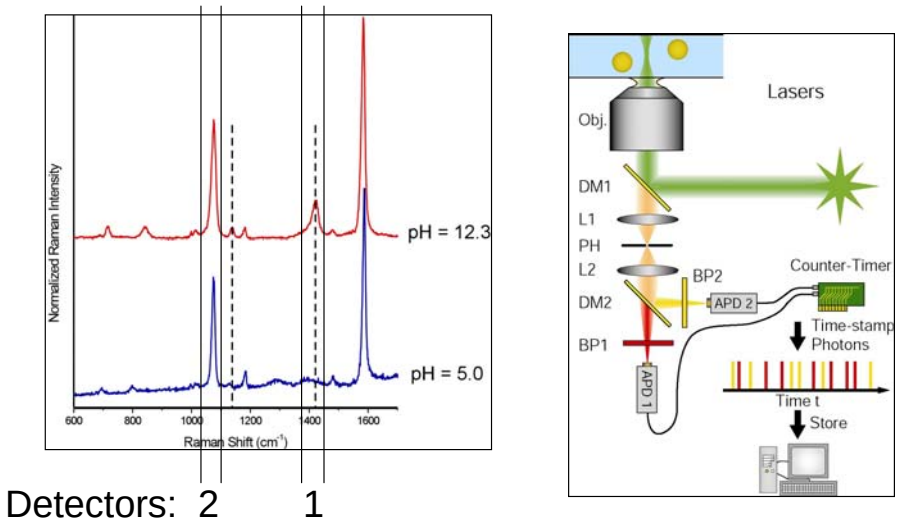

Figure 3: To measure pH using SERS, we select a Raman line that varies with pH (detector 1; selected using bandpass filter BP1) and on a Raman line that is constant (detector 2; selected using BP2). Confocal fluorescence microscopy is used to detect scattering particles; fast avalanche photodiodes (APD1 and APD2) are used to detect the scattered photons.

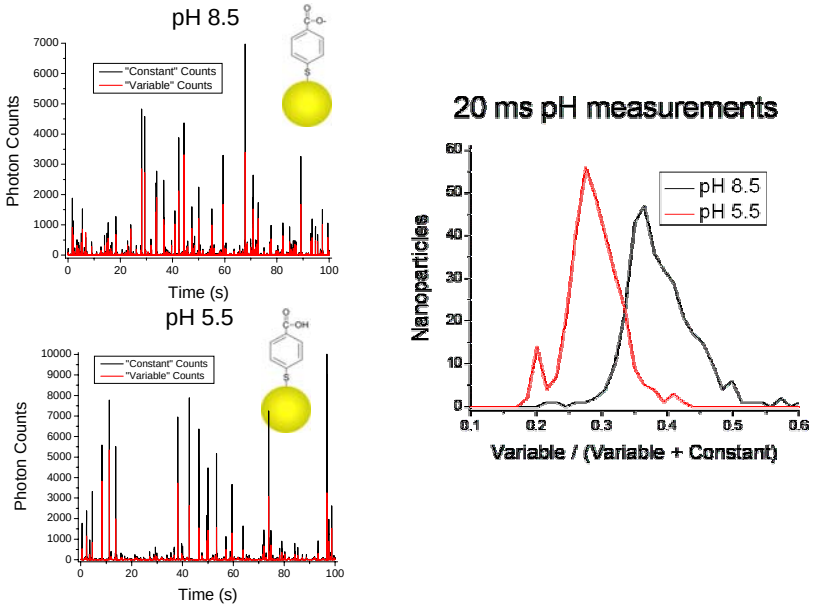

Figure 4: Photon burst data for silver nanoparticles in pH 8.5 buffer (upper left) and pH 5.5 buffer (lower left). On the right is the histogram of detected nanoparticles, where the x axis is the ratio of the pH dependent line to the sum of the pH dependent and pH independent lines.

Characterization of signal intensity fluctuations.

Previous SERS nanoparticle studies have focused on spectroscopy, and, consequently, fluctuations of signals on sub-millisecond time scales are poorly characterized. The methodology we developed for performing rapid pH measurements also allows characterization of these faster fluctuations. By looking at correlation functions derived from the fluctuating signals, we are able to observe diffusion processes. The second figure shows a correlation function with two observed diffusion processes: translational and rotational diffusion. The visibility of the rotational diffusion process is polarization dependent, indicating a dipole nature to the scattering particles (or clusters).

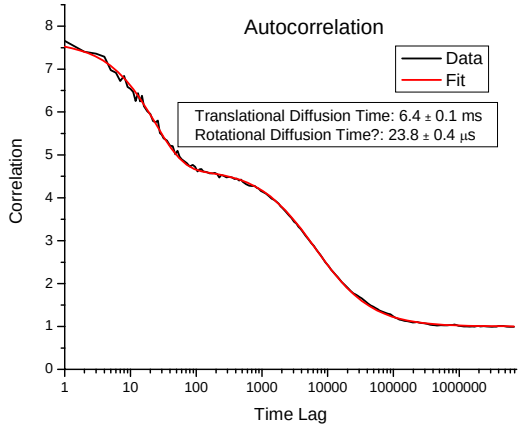

Figure 5: Correlations calculated from intensity measurements of diffusing nanoparticles reveal processes with two time scales: the longer timescale corresponds to translational diffusion of the nanoparticles across the confocal excitation, the shorter timescale corresponds to rotational diffusion of the nanoparticles with respect to the polarization of the excitation laser.

CONCLUSIONS

A new type of optical probe, based on monitoring the SERS response of functionalized metal nanoparticles has been developed. The application of SERS correlation spectroscopy of specific peaks in the Raman spectrum of the functionalized probes was presented as a means to enable rapid probing of changes in the local pH. We have demonstrated that these probes have optical properties, i.e. high photostability and signal intensities that are superior to fluorescent probes. Currently, the pH range of these probes, however, is limited by their tendency to form clusters, which results in a complex pH microenvironment between particle junctions due to variations in particle dimensions. We are currently developing approaches to control particle heterogeneity which will allow us to overcome these limitations. A future application of these nanoscales optical probes will be the measurement of intra- and extracellular pH in neuroblastoma cells lines that are known to have a large pH gradient across the cell membrane.

ACKNOWLEDGEMENTS

Funding for this work was provided by the Laboratory Directed Research and Development Program at Lawrence Livermore National Laboratory and the Genomics:GtL Program of the Office of Science of the U.S. Department of Energy. This work has also been supported by funding from the National Science Foundation. The Center for Biophotonics, an NSF Science and Technology Center, is managed by the University of California, Davis, under Cooperative Agreement No. PHY 0120999. This work was performed under the auspices of the U.S. Department of Energy by University of California Lawrence Livermore National Laboratory under contract No. W-7405-Eng-48.

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